EXHIBIT 2

into an exon or intron of an actively transcribed endogenous gene, thereby simultaneously disrupting the gene and acting as a locus-specific marker of the gene. This would greatly facilitate the identification and cloning of the disrupted genes, a task that is not necessarily straightforward following chemical mutagenesis. In practice, hundreds of transgenic embryos, each carrying multiple integration sites, could be generated per day and screened for GFP expression. A huge advantage of using this approach in frog embryos is that they develop externally, therefore GFP expression can be assayed in living embryos at any stage. Most embryos will not express the marker gene. These will be discarded and only the few that express will be nurtured to maturity, thus greatly reducing the number of embryos that must be carried to the next generation. Preliminary experiments in *X. laevis* strongly suggest that using a gene trap approach will be productive (O. Bronchain and E. Amaya, unpublished).

The powerful manipulations that one can perform on amphibian embryos have been used to reveal important principles about development for over a century. As we approach the next century, it appears that it will now be possible to overlay this rich embryological history with the power of genetic manipulations, creating an armamentarium of approaches as we look towards revealing a new generation of concepts about vertebrate embryonic development.

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Double-stranded RNA as a mediator in sequence-specific genetic silencing and co-suppression

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Many fundamental natural processes have been uncovered not by preplanned scientific enquiry, but serendipitously by engineers and scientists who observed unexpected consequences of their manipulations. Biologists routinely use engineering to manipulate the expression of specific genes and, thus, understand (or benefit from) their function. Sometimes we wish to make a particular gene silent; at other times we want the genes to 'talk' more loudly. Attempts at silencing have often employed an antisense strategy of introducing single-stranded nucleic acid from the noncoding strand to sequester or modify the native transcript, thereby preventing accumulation of the corresponding protein. Conversely, by introducing extra copies of a specific gene, one might expect in many cases to overproduce the corresponding mRNA and protein products. Although these techniques have been successful in numerous applications, a body of literature is emerging that documents certain cases in which unexpected outcomes of these manipulations are seen in organisms as diverse as nematodes and plants. These observations encompass 'transgene silencing' (a failure to express certain multicopy transgenes) and co-suppression (the ability of a 'sense' transgene to interfere with the activity of the endogenous genetic locus). Certain of these phenomena are thought to involve direct DNA–DNA interactions, whereas others have been proposed to require an RNA effector molecule. The structure and mechanistic properties of RNAs mediating the latter type of co-suppression have yet to be elucidated. Here, we discuss the possibility that double-stranded RNA (dsRNA), rather than sense or antisense single-stranded RNAs alone, is the effector molecule responsible for RNA-mediated silencing and co-suppression.

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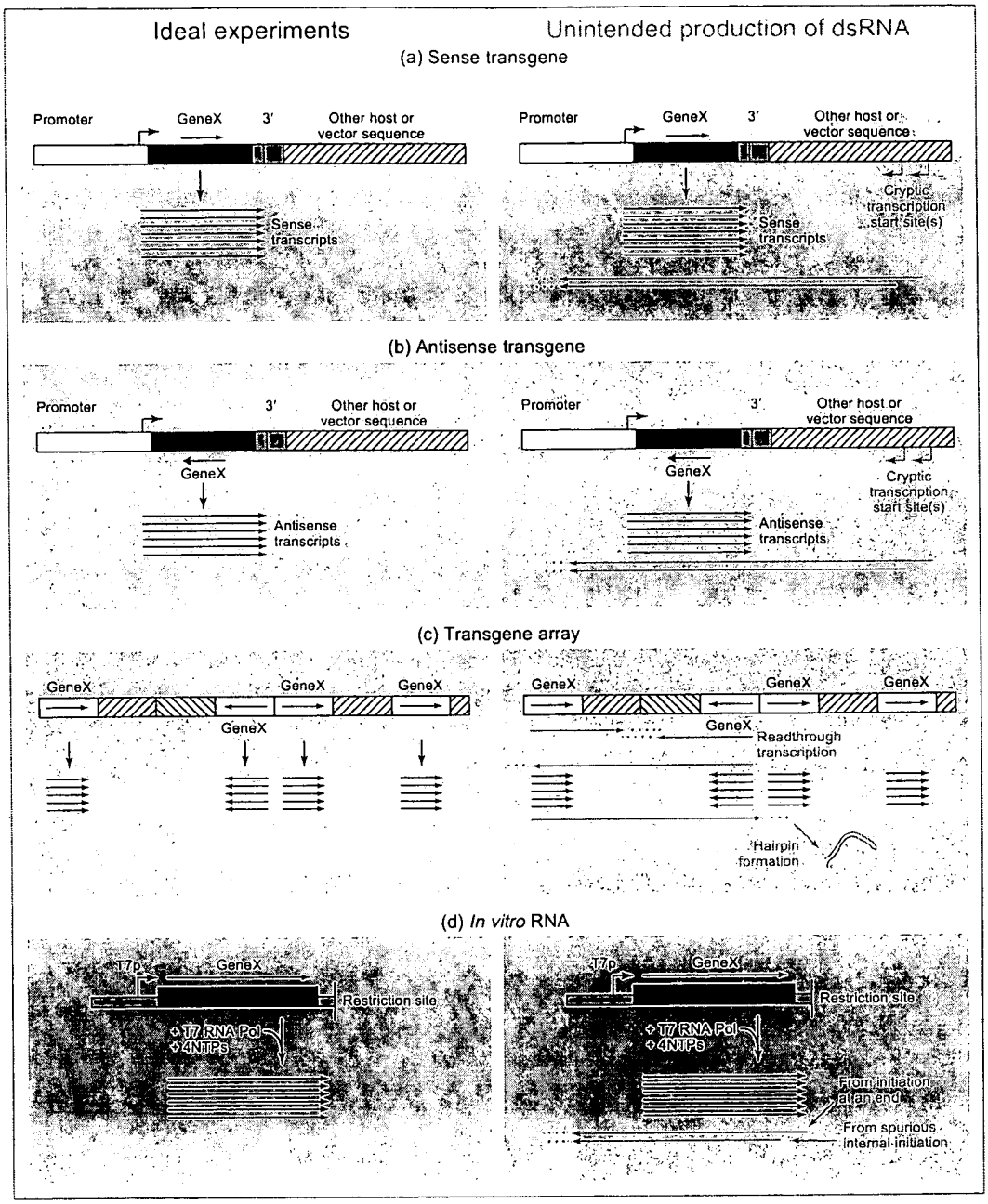

FIGURE 1. Unintended production of double-stranded (ds) RNA. Left, a series of theoretical experiments that might be designed to produce a pure population of single-stranded RNA. Right, how a low level of dsRNA could also be produced in each case. (a) A transgene designed to produce 'sense' RNA is transcribed at low level from a cryptic (or natural) start site on the opposite strand. Hybridization to 'sense' transcripts from the same template would result in dsRNA. (b) A transgene designed to produce 'antisense' could similarly be subject to low-level transcription on the opposite strand, with dsRNA resulting from hybridization of the newly transcribed RNAs. (Note that antisense RNA might alternatively hybridize with the endogenous chromosomal transcript to make dsRNA; it is not clear, however, that sense and antisense RNAs sythesized at distant nuclear sites would form dsRNA and be capable of interference.) (c) A transgene array containing tandem and inverted copies of a DNA construct ('geneX') might be expected to produce only one strand of RNA. Note, however, that readthrough of the geneX terminator would produce RNA with an inverted repeat structure. This RNA could undergo intramolecular hybridization to produce a predominantly double-stranded hairpin. (d) During *in vitro* synthesis of RNA, transcription initiates primarily at the bacteriophage RNA-polymerase promoter. Initiation can, however, also occur at internal sites and template ends, which leads to some inclusion of dsRNA in 'sense' and 'antisense' RNA preparations.

RNA-mediated genetic interference (RNAi) in the nematode *C. elegans*

Several years ago it was reported that antisense RNA targeted to specific endogenous genes in *Caenorhabditis elegans*, when either expressed from a transgene[1] or injected directly into the worm's gonad[2], could phenocopy a null or hypomorphic mutation in the targeted gene. Surprisingly, both reports indicated that sense transcripts also were effective in producing the targeted phenotype. These observations were difficult to explain using a conventional model of antisense sequences inactivating the sense transcript. Recently, at least part of this mystery was solved by the discovery that much, if not all, of the genetic interference from injected 'sense' and 'antisense' RNA is actually mediated by double-stranded RNA (dsRNA) that is present at a low level in all *in vitro* RNA syntheses because of the non-specific activity of RNA polymerases[3] (Fig. 1). Highly purified preparations of antisense (and sense) RNAs had negligible effects, whereas dsRNA exhibited potent and specific interference against any of a variety of targeted genes. The potency of RNAi in worms points to the existence of novel mechanism(s)[3]. Even with an abundantly transcribed target (several thousand mRNA copies per cell), a few molecules of dsRNA per cell can produce specific inhibition. This would not be expected from a simple antisense mechanism; there is simply not enough material to bind to all the endogenous mRNA targeted for destruction. These results generated two fundamental questions: how can dsRNA mediate gene-specific interference; and what is the physiological purpose of this process?

RNA-mediated silencing and co-suppression in plants

A second body of work on RNA-mediated interference comes from the plant world[4]. In the late 1980s, plant researchers were surprised to find that the introduction of certain transgenes into plants can result in homology-dependent silencing of an endogenous locus (rather than over-expression of the coding region of interest). This phenomenon is referred to as co-suppression. Not all transgenes cause this effect; there is no current basis for predicting which would and which would not. Gene silencing in plants has been proposed to encompass a variety of different mechanisms[4,5], including some that act by direct DNA–DNA interaction and others that involve interference by an RNA product of the transgene. Strong evidence for the latter class of mechanisms comes from experiments in which RNAs are introduced in the absence of a DNA template (using RNA viruses as vectors); the ability of viral RNAs to interfere with a homologous gene in the plant genome is one of the strongest arguments for the existence of RNA-mediated silencing mechanisms[6–8].

The literature contains a few clues as to the nature of the interfering RNA. In certain cases, co-suppression is correlated with high-level transcription of the transgene[9,10]. Given recent results in *C. elegans*, we pose the possibility that transcription along the antisense strand of a transgene could result in low levels of interfering dsRNA (Fig. 1). Such transcription might be low-level synthesis directed by sequences within the vector or flanking regions at the site of integration (e.g. see Ref. 11). Significantly, Que *et al.*[10] reported that, whereas co-suppression was associated with accumulation of transcripts at high concentrations from single-copy transgenes, inversely repeated transgenes could cause co-suppression, irrespective of promoter strength or level of the transgene mRNA. Transcripts from inversely repeated transgenes would be expected to produce a double-stranded structure. Experiments with chimeric RNA viruses[7,8] might similarly point toward a dsRNA involvement; in these experiments, the viral RNA replicase copies the chimeric RNA in the cytoplasm, generating both sense and antisense material.

Similarities between nematodes and plants

RNAi in worms and co-suppression in plants share some striking similarities. Both are cases of gene-specific interference. dsRNA has been shown to be the agent of interference in nematodes and, as suggested above, there is some indication that dsRNA could also be responsible for co-suppression in plants. Perhaps the most interesting common characteristic is that the phenomenon can spread from the site of interfering RNA synthesis or application. In worms, the dsRNA mix can be injected into the body cavity, where it can produce an interfering effect in distant tissues and in F1 progeny, indicating that cells may have an RNA-transport mechanism[3]. Similarly, two groups of researchers have demonstrated the systemic spread of co-suppression in plants[12,13]. An RNA molecule, spreading throughout the plant via phloem, has been proposed as the mobile agent responsible for transmitting the co-suppression state[12].

Possible mechanisms for RNA-mediated interference

The sub-stoichiometric activity of the interfering RNA in *C. elegans* led to various models: that interference involves a catalytic mechanism dependent on the injected RNA; that the input material is amplified; or that interference occurs at the level of the gene. Several lines of evidence argue against DNA in the genome as a target for RNAi. Effects of dsRNA are generally not heritable beyond the first generation; injected animals and progeny exhibit the effects of RNAi, whereas animals of the F2 generation generally revert to a wildtype phenotype[3]. Additional evidence comes from direct sequencing of genomic DNA following RNA-mediated interference with *unc-22*; these experiments yielded no indication of mutations in the target gene (S. Xu and A. Fire, unpublished). Consistent with an RNA target, interference was effective using a variety of regions present in mature RNA, but was not effective using intronic or promoter sequences[3]. At this point, one attractive hypothesis is that dsRNA might result in early degradation of the endogenous mRNA. We know from *in situ* hybridization studies that RNA transcripts of a target gene fail to accumulate after RNAi (Ref. 3). Conceivably, the lack of mRNA products could be an indirect consequence of blocked processing or transport. Alternatively, endogenous transcripts could be degraded by a sequence-specific mechanism directed by dsRNA.

The mechanisms mediating certain co-suppression phenomena in plants have been shown to act both on the DNA template and on RNA products. Wassenegger *et al.*[6] showed that viral or transgene-generated RNA could direct *de novo* modification (presumably methylation) of a homologous sequence in the plant genome. Other studies provide cases in which RNA-mediated co-suppression acts

post-transcriptionally, potentially by the rapid degradation of the target transcript[14]. In one example, transgene-mediated silencing of the endogenous gene encoding β-1,3-glucanase in tobacco, de Carvalho Niebel and colleagues[15] demonstrated that the suppressed genes are actively transcribed. Subsequently, Jacobs *et al.*[16] showed that gene silencing in this line correlates with an increased turnover of both the transgenic and the endogenous transcripts of β-1,3-glucanase. In the case of virally provided RNA sequences, it appears that viral RNA molecules can serve as targets as well as 'triggers' for co-suppression[8,13,17]. Could there be a mechanistic link between (a) RNA-mediated degradation of RNA and (b) RNA-mediated methylation of DNA? One possibility is that these are two separate processes mediated by similar RNA molecules; alternatively, there could be a causal relationship, perhaps from an ability of RNA decay products to trigger methylation of homologous sequences in replicating DNA.

Does RNA-mediated interference do a job for the cell?

In addition to the mechanistic questions, attention is also merited to the physiological role for the RNA-associated silencing phenomenon. A role for co-suppression mechanisms in systemic defense against viruses has been suggested for plants[17,18] and could apply to other organisms as well. Such a response represents an effective means by which to prevent viral replication and induce resistance in surrounding tissues prior to viral invasion.

Alternatively, co-suppression/RNAi might modulate normal gene expression. One can easily imagine double-stranded RNAs being used by the cell as a potent means to turn off specific genes in response to physiological or developmental cues. Perhaps the best way to identify these processes will be to find mutants that are defective in carrying out RNAi.

Do RNA-interference mechanisms have counterparts outside of plants and nematodes?

Mammalian cells exhibit a global antiviral response to double-stranded RNA. In this response, the PKR protein kinase recognizes dsRNA and unleashes a vehement but somewhat non-specific response leading to general translational arrest[19]. Intriguingly, this type of systemic response can occur if the dsRNA is provided extracellularly[20] (consistent with the possibility of dsRNA uptake by mammalian cells). Viruses have evolved a number of strategies for evading or inhibiting the PKR response[21]. Certain tissue-culture cell lines lack PKR and are susceptible to mutant viruses that would otherwise be non-virulent. Any gene-specific interference by dsRNA in PKR-proficient mammalian cells would be dependent on a transient lapse in the PKR response, or on a controlled level of dsRNA that was incapable of activating PKR.

A wealth of information indicates that specific RNA-mediated interference mechanisms contribute to the control of gene expression in vertebrate and other systems. For many of these contributions, the precise nature of the interfering RNA (single-stranded versus double-stranded material) has yet to be characterized. Antisense transcripts have been reported for large numbers of vertebrate genes[22]. In some cases, roles for these transcripts in regulating the sense transcripts from the opposite strand have been demonstrated. From an informatics perspective, a surprisingly large fraction of vertebrate mRNAs contain long-conserved sequences within the 3′ untranslated region as well as long blocks without silent changes in their protein-coding regions[22]. Lipman[22] has proposed that these conserved sites are regulatory targets of endogenous antisense transcripts encoded by the complementary strand of the gene. Such a mechanism would, thus, be common and relatively conserved. Endogenous genes regulated by antisense transcripts have also been described for the primitive eukaryote *Dictyostelium*, and such mechanisms have been studied in detail in Eubacteria and Archaebacteria (reviewed in Ref. 23). Co-suppression phenomena, similar to that described for plants, have also been observed in *Dictyostelium*[24]. It will be interesting in the next few years to learn whether any or all of these effects share underlying mechanistic features and we suggest, moreover, that by studying the mechanisms underlying these phenomena, we will be better able to interpret the native language of the cell.

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